



# Cytokine release and adhesion molecule expression by stimulated human bronchial epithelial cells are downregulated by salmeterol<sup>☆</sup>

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## KEYWORDS

Asthma;  
Adhesion molecules;  
Cytokine release;  
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 $\beta_2$ -agonists

**Summary**  $\beta_2$ -adrenoreceptor agonists are able to modulate various aspects of airway cell functions involved in the inflammatory and repair processes characterizing a variety of respiratory disorders. Human bronchial epithelial cells (HBECs), which can act as immune effector cells and express  $\beta_2$ -adrenoreceptors, were used to test the effects of different concentrations (0.1–100.0 nM) of salmeterol (*Salm*) on adhesion molecule expression and chemokine/cytokine release. HBECs, freshly isolated from resected bronchi at the time of surgery in ex-smokers with lung cancer, constitutively expressed over 3 times more ICAM-1 than VCAM-1 ( $P < 0.05$ ) and secreted greater amounts of IL-8 than of GM-CSF or RANTES ( $P < 0.001$ ). Stimulation of HBECs with IL-4, TNF- $\alpha$  or IL-4 plus TNF- $\alpha$  upregulated ICAM-1 expression ( $P < 0.05$ ) and increased GM-CSF and IL-8 secretion ( $P < 0.05$ ). Similarly, VCAM-1 expression was significantly increased by IL-4 plus TNF- $\alpha$ , while RANTES release was significantly enhanced by IL-4 or by IL-4 plus TNF- $\alpha$  ( $P < 0.05$ ), but not by TNF- $\alpha$  alone ( $P > 0.05$ ). Dose-response curves showed that *Salm*, at concentration  $> 1.0$  nM, was effective in inhibiting adhesion molecule expression and cytokine release by HBECs ( $P < 0.05$ ). At a *Salm* concentration of 10 nM the degree of inhibition observed was similar for ICAM-1 and VCAM-1 expression ( $37.2 \pm 9.3\%$  and  $32.9 \pm 9.6\%$ , respectively;  $P > 0.05$ ), but higher for RANTES ( $88.4 \pm 4.4\%$ ), as compared to IL-8 ( $21.8 \pm 7.0\%$ ) or GM-CSF ( $30.1 \pm 6.6\%$ ;  $P < 0.05$ , each comparison). Thus, adhesion molecules and cytokines may be expressed/released at very different levels by unstimulated or stimulated HBECs and those activities appear to be modulated by *Salm*.

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## Introduction

Besides being a physical and functional barrier to external agents, bronchial epithelial cells appear to be involved in the pathogenesis of the inflam-

matory and repair processes that characterize a variety of respiratory disorders.<sup>1</sup> Indeed, airway epithelial cells express adhesion molecules involved in cell–cell and cell–extracellular matrix interaction and are source of mediators capable of sustaining inflammation and facilitating repair and remodelling processes that follow injury.<sup>1,2</sup> These considerations have lead to the concept that these cells should be considered a major target for therapeutic intervention.<sup>1–3</sup>

$\beta_2$ -adrenoreceptor activity is a key part of the physiological and pharmacological response in

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subjects with chronic obstructive lung diseases.<sup>4</sup> Indeed, bronchial smooth muscle relaxation results directly from  $\beta_2$ -adrenoreceptor stimulation.<sup>5</sup> However, since these receptors have been identified on many cells in the lung, including bronchial epithelial cells, it should be expected that  $\beta_2$ -adrenoreceptor agonists might mediate a variety of other functions through the stimulation of  $\beta_2$ -adrenergic receptors expressed on other cell types.<sup>6</sup> In this context, these drugs may inhibit plasma exudation acting on postcapillary venule endothelial cells and effectively downregulate the release of bronchoconstrictor substances from mast cells and of proinflammatory mediators from eosinophils, neutrophils, macrophages, and T-cells.<sup>7-10</sup> By contrast, the effect of  $\beta_2$ -agonists on airway epithelial cell functions is uncertain and sometimes contradictory.<sup>6</sup> While it is established that at least in vitro  $\beta_2$ -adrenoreceptors may increase ciliary beat frequency,<sup>1,11,12</sup> cell proliferation,<sup>13</sup> heat shock proteins<sup>14</sup> and monocyte adhesion to airway epithelium via cAMP-mediated changes in epithelial cells,<sup>15</sup> less clear is their activity on bronchial epithelial cell pro-inflammatory functions. Indeed, while with short-acting  $\beta_2$ -adrenoreceptor agonists inhibition of adhesion molecule expression<sup>16</sup> and no effect or downregulation of IL-8 and GM-CSF release have been reported,<sup>17,18</sup> inhibition of GM-CSF associated with increase production of IL-8 were observed when exposing human airway epithelial cells to the long-acting  $\beta_2$ -adrenoreceptor agonist formoterol.<sup>19</sup> Discrepancies in the results may be related to differences in the source of cells, experimental conditions cell types, on the cell functions evaluated and on the characteristics of the compound tested.<sup>4-6,20-22</sup>

With this background a study was designed to test in vitro the activity of another long-acting  $\beta_2$ -adrenoreceptor agonist widely used in the treatment of patients with asthma and other chronic obstructive lung diseases, salmeterol, on freshly isolated human bronchial epithelial cells. The cells were stimulated with IL-4 and TNF- $\alpha$ , i.e. two cytokines released in the local milieu by other cells in inflammatory airway disorders<sup>3</sup> and effective in stimulating bronchial epithelial cells.<sup>1,23,24</sup> The activity of salmeterol was tested on two different epithelial cell functions, i.e. adhesion molecule (ICAM-1 and VCAM-1) expression and cytokine (IL-8, GM-CSF and RANTES) release. Among the variety of surface molecules expressed on the surface of the bronchial epithelium, ICAM-1 and VCAM-1 were selected because of their importance in the interaction between airway epithelial cells and inflammatory cells.<sup>25</sup> Indeed, ICAM-1 is the ligand for surface molecules expressed on eosinophils,

monocytes and lymphocytes and its expression is increased at sites of inflammatory reactions and is associated with leukocyte transmigration, adhesion and activation.<sup>25-27</sup> VCAM-1 acts as ligand for VLA-4, an adhesion molecule expressed by monocytes, lymphocytes and eosinophils, and is engaged in eosinophil activation and survival.<sup>25,28,29</sup> The activity of salmeterol was also tested on the secretion of three proinflammatory cytokines/chemokines IL-8, GM-CSF and RANTES. IL-8, recognized as neutrophil chemoattractant and activator, is also able to induce T-lymphocyte migration and to enhance eosinophil locomotion,<sup>1,2,30</sup> while GM-CSF is one of the major "eosinophilic" cytokine being involved in the maturation, recruitment, activation and survival of these cells.<sup>31</sup> Finally RANTES, a cytokine active on monocytes and activated memory T cells, has also been shown to be a potent chemoattractant for eosinophils and an efficient basophil histamine-releasing factor.<sup>32,33</sup>

The results showed that resting or stimulated airway epithelial cells may express/release adhesion molecules and cytokines at very different levels and that salmeterol is effective in down-regulating these biological functions.

## Materials and Methods

### Isolation and culture of human bronchial epithelial cells (HBECs)

HBECs were isolated and cultured as previously described.<sup>21</sup> Briefly, human bronchi, obtained from eight individuals at the time of surgical procedures for localized lung tumours. The donors were not treated with inhaled glucocorticosteroids or  $\beta_2$ -adrenoreceptor agonists at the time of surgery. The bronchi, histologically normal, were trimmed off the lung parenchyma and peribronchial vascular structures, and incubated at 4°C overnight in sterile calcium-free Eagle's MEM (ICN Biomedicals Inc.) containing 0.1% bacterial protease type XIV (Sigma-Aldrich, Milan, Italy). Bronchial lumina were then gently rinsed several times using a syringe containing MEM plus 10% FCS to detach the HBECs. The collected cells were washed once in the same medium, filtered through one layer of surgical sterile gauze and washed twice with MEM. The cell count was manually determined using a standard hemocytometer. Extensive evaluation of cells prepared with this method showed that >95% of the harvested cells were HBECs (cytochrome positive). HBECs were plated for growth at 37°C in 5% CO<sub>2</sub> in tissue culture plates (Costar

Corp., Cambridge, MA, USA) previously coated with murine type I collagen. LHC basal medium (Biofluids Inc., Rockville, MD, USA) was used, supplemented with 5 mg/ml insulin, 5 mg/ml transferrin, 5 mM phosphoethanolamine/ethanolamine, 10 nM T3, 0.33 nM retinoic acid, trace elements, 0.11 mM calcium, bovine pituitary extract containing 10 mg/ml protein (0.5%), 50 mg/ml penicillin-streptomycin, and 2 mg/ml fungizone.<sup>21</sup> The LHC-supplemented medium was mixed 1:1 with RPMI 1640, a solution found to optimize HBEC growth. In the average cell preparation, 20–30% of the viable cells plated at the start of the culture were attached to the tissue culture plates after 24 hours and about a quarter of the adherent cells were able to proliferate. Since the confluent HBECs tend to assume a squamous shape when replated and are less responsive to the stimuli, the growing HBECs were maintained in a subconfluent state by replating the cells every 2–3 days.

### Cells stimulation and evaluation of the effects of salmeterol on HBEC functions

After being seeded in 24-well tissue plates (70,000 cells well<sup>-1</sup>), previously coated with bovine type I collagen, HBECs were cultured for 24 h and stimulated with interleukin (IL)-4 (20 ng ml<sup>-1</sup>, 1.34  $\mu$ M, final concentration,) and/or tumour necrosis factor (TNF)- $\alpha$  (20 ng ml<sup>-1</sup>, 1.18  $\mu$ M, final concentration) or, as control, with medium alone.<sup>16</sup>

Indeed, in preliminary sets of experiments, these IL-4 and TNF- $\alpha$  concentrations were effective in inducing an upregulation of the different HBEC functions tested. To evaluate the activity of the drug, HBEC cultures were stimulated for 24 h with IL-4 combined with TNF- $\alpha$  in the presence of different concentrations of salmeterol (0.1–100.0 nM). In all the experimental conditions, after a 24 h incubation, HBECs were trypsinized, washed, resuspended in the appropriate medium, and molecule adhesion expression and cytokine release were evaluated as described below.

### Evaluation of adhesion molecule expression on HBECs

To evaluate the expression of ICAM-1 (CD54) or VCAM-1 (CD 106), 100  $\mu$ L of the cell suspensions were placed into round-bottom microtiter 96-well plate (Costar Corp), incubated for 30 min at 4°C with a monoclonal antibody (mAb) anti-human (ah)-CD54 (Caltag Laboratories, Burlingame, CA) or ah-CD106 (Southern Biotechnologies Associates, Inc. Birmingham, USA) fluorescein isothiocyanate

(FITC) conjugated (Caltag Laboratories, Burlingame, CA), washed twice and then fixed with 0.5% paraformaldehyde.<sup>16</sup> The cells were analyzed by single color immunofluorescence flow cytometry (Becton Dickinson Immunocytometry Systems; Mountain View, CA, USA). To compare the fluorescence intensities of different samples from the same experiments, the analysis were performed with identical settings of the logarithmic amplifier and listmode files were analysed with CELLQuest software (Becton Dickinson). After conversion to linear fluorescence intensity units to obtain a linear function of fluorescence intensity over a wide range, the average background linear fluorescence obtained with the control antibody, the anti-human (ah)-CD3 FITC, was subtracted from the average fluorescence intensity of the specifically stained cells. The possible, changes in cell size were controlled by means of the forward light scatter signal of the flow cytometer, proportional to cell size and algebraically adjusting for increases in cell surface area so that the intensity in relative linear fluorescence units could be obtained.<sup>16</sup> This correction permits to directly correlate the average fluorescence intensity with the cell surface density of the stained antigens. All experiments were performed in triplicate. The intensity of fluorescence was expressed as mean fluorescence channel (mfc).<sup>16</sup>

### Evaluation of cytokine release by HBECs

Culture supernatants from HBEC cultures were tested for the presence of IL-8, GM-CSF, and RANTES. Concentrations of RANTES in the cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (Amersham International) according to manufacture's instructions, while IL-8 and GM-CSF were measured by a sensitive, novel cytometric technique, Quanti Flow TM IFA System, (BioErgonomics, Inc., White Bear Lake, MN).<sup>22</sup> Briefly, for the last system, the samples were incubated with cytokine capture beads for 1 h at room temperature with mixing (nutation). After washing, the samples were incubated with a fluorescein-labelled reporter antibody (10  $\mu$ L) for 30 min at room temperature with nutation. After washing, the samples were resuspended in PBS and analyzed by flow cytometry. Concentration of secreted cytokine was determined by comparing mean fluorescence channel of the sample to standard curve. The standard curve was developed by plotting the mean fluorescence channel of each standard vs. the concentration of the cytokine on a log vs. log graph.<sup>22</sup>

## Statistical analysis

Statistical evaluation was performed on a Power Macintosh G3 (Apple Computer, Cupertino, CA, USA) using the statistical software package Statview II (Brainpower Inc., Calabasas, CA, USA). All data are expressed as arithmetic mean  $\pm$  standard error of the mean. Paired *t*-test, unpaired *t*-test or Mann–Whitney *U*-test was used when appropriate. The mean values were considered to be statistically significant when the probability of the event was below 5% ( $P < 0.05$ ).

## Results

### Characteristics of human bronchial epithelial cell (HBEC) cultures

Number, differential count and viability of the cell collected by scraping surgically resected bronchi were evaluated. The total number of HBECs recovered was  $18.2 \pm 2.2 \times 10^6$  cells. The percentage of cells identified as HBECs on cytocentrifuge preparations were  $99.4 \pm 0.3\%$ ; only a small proportion of macrophages ( $0.3 \pm 0.2\%$ ), lymphocytes ( $0.2 \pm 0.1\%$ ), polymorphonuclear leukocytes ( $0.3 \pm 0.3\%$ ) contaminated the samples collected (not shown). Finally, the proportion of viable HBECs, i.e. excluding trypan blue dye, was consistently elevated in the different experiments ( $87.2 \pm 7.6\%$ ).

### Constitutive and IL-4 and/or TNF- $\alpha$ -induced adhesion molecule expression and cytokine release

Evaluating adhesion molecule expression on unstimulated HBECs growing in LHC9 medium, we found that the constitutive expression of ICAM-1 was higher than that of VCAM-1 ( $P < 0.05$ ) (Fig. 1A). Stimulation of the cells with IL-4 or TNF- $\alpha$  or with the combination of IL-4 plus TNF- $\alpha$ , induced an increase in ICAM-1 expression ( $P < 0.05$ , each comparison with unstimulated cells) (Fig. 1B). In contrast, only the combination of IL-4 plus TNF- $\alpha$  enhanced VCAM-1 expression ( $P < 0.01$ , each comparison) (Fig. 1C).

HBECs constitutively secreted detectable amounts of IL-8, GM-CSF and RANTES. However, the IL-8 levels in the supernatants were over 200-fold higher than those of GM-CSF and over 3500-fold higher than those of RANTES ( $P < 0.001$ , each comparison) (Fig. 2A–C). While incubation with IL-4 or TNF- $\alpha$  alone induced a significant increase in the

release of IL-8 and GM-CSF ( $P < 0.05$ , each comparison with unstimulated cells), RANTES secretion was increased only in cultures stimulated by IL-4 ( $P < 0.05$ , comparison with unstimulated cells). Interestingly, the combination of IL-4 plus TNF- $\alpha$  induced a statistically significant increase in the release of RANTES and GM-CSF, as compared to IL-4 or TNF- $\alpha$  alone, and in the release of IL-8, as compared TNF- $\alpha$  alone (Fig. 2A–C). The magnitude of the increase in IL-8, GM-CSF and RANTES production by stimulated epithelial cells was not related to the constitutive levels of the same cytokines ( $P > 0.05$ , each correlation, not shown).

### Effects of salmeterol on IL-4 + TNF- $\alpha$ -induced HBEC adhesion molecule expression and cytokine release

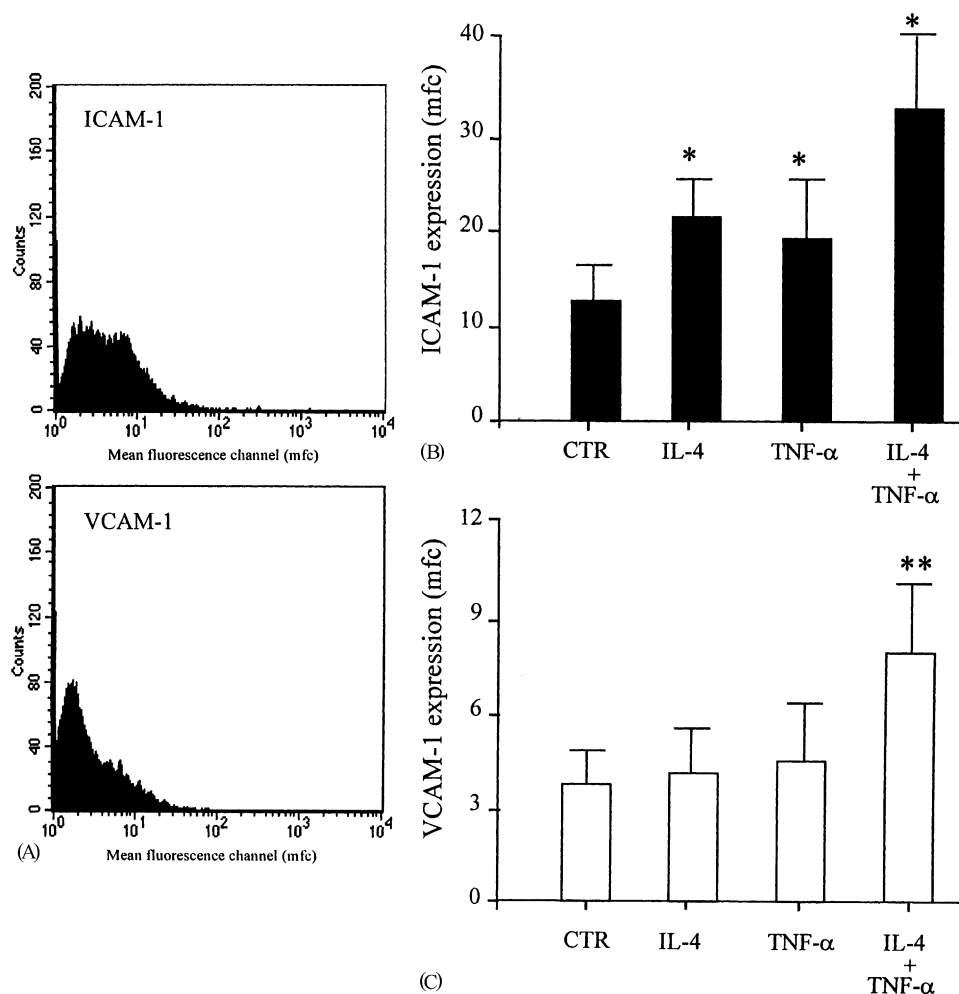
Since neither IL-4 nor TNF- $\alpha$  alone significantly modified VCAM-1 expression, the effects of salmeterol on adhesion molecule expression and cytokine release were evaluated on HBECs stimulated by IL-4 plus TNF- $\alpha$ .

Independently from the constitutive or the induced levels of adhesion molecule expression, the addition of salmeterol to the culture medium induced a dose-dependent inhibition of the expression of ICAM-1 and VCAM-1 (Fig. 3) induced by IL-4 + TNF- $\alpha$ , being significant (as compared to untreated cells) at the two highest concentrations tested ( $P < 0.05$ , each comparison). Similar results was obtained when cytokine release were evaluated. Salmeterol was effective in downregulating IL-8, GM-CSF and RANTES release (Fig. 4) in a dose-dependent fashion, the inhibition being significant (as compared to untreated cells) at the two highest concentrations tested ( $P < 0.05$ , each comparison).

The degree of inhibition was similar for ICAM-1 and VCAM-1 expression ( $P > 0.05$ ; each comparisons) but higher for RANTES, as compared to IL-8, GM-CSF ( $P < 0.05$ ; each comparison).

## Discussion

Working on cells, freshly isolated from surgically resected bronchi, we have shown that: (a) bronchial epithelial cells can express, constitutively and upon activation, adhesion molecules, such as ICAM-1 and VCAM-1, and secrete proinflammatory proteins, such as IL-8, GM-CSF and RANTES; (b) salmeterol, a long acting  $\beta_2$ -adrenergic drug, is effective in vitro in inhibiting adhesion molecule expression and chemokine/cytokine release by stimulated epithelial cells.



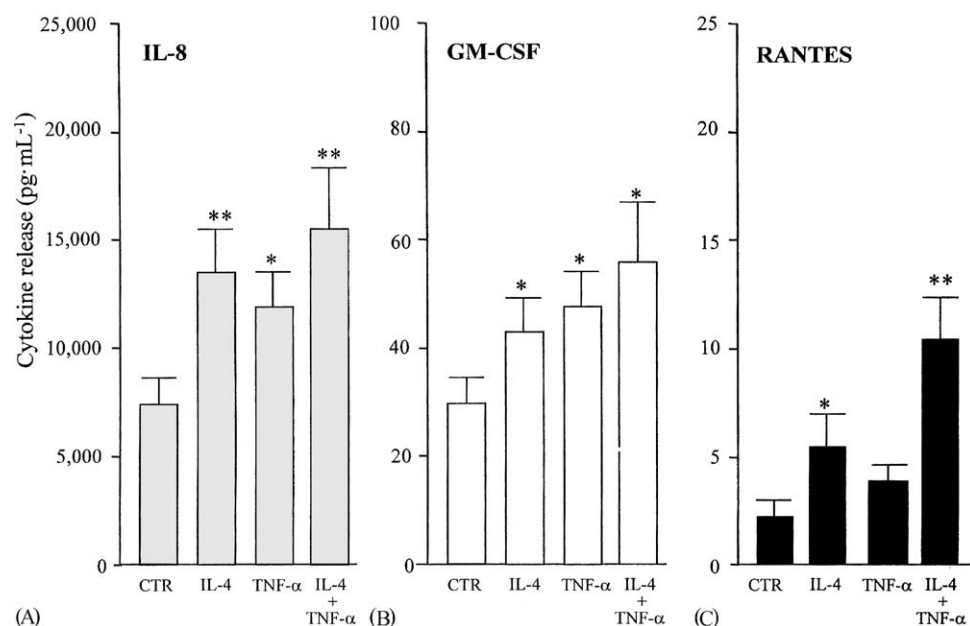
**Figure 1** Constitutive and cytokine-induced adhesion molecule ICAM-1 and VCAM-1 expression by human bronchial epithelial cells. (A) Flow cytometric histograms of constitutive ICAM-1 and VCAM-1 expression by unstimulated HBECS. Cell number is shown on the ordinate, while the green fluorescence intensity given by anti-human ICAM-1 or anti-human VCAM-1 FITC-conjugated monoclonal antibody is shown on the abscissa as mean fluorescence channel (mfc). (B and C) Changes of ICAM-1 expression (B) or VCAM-1 expression (C) on the surface of HBECS induced by IL-4 (20 ng·ml<sup>-1</sup>), TNF- $\alpha$  (20 ng·ml<sup>-1</sup>), or IL-4 + TNF- $\alpha$  (20 ng·ml<sup>-1</sup>). The intensity of adhesion molecule expression (mean fluorescence channel (mfc)) is shown on the ordinate, whereas the different culture conditions are shown on the abscissa. The data are expressed as mean  $\pm$  SEM, and represent the results of eight experiments \* $P$  < 0.05; \*\* $P$  < 0.01; as compared to unstimulated cell cultures.

In the experimental conditions here reported, ICAM-1 and VCAM-1 expression and IL-8, GM-CSF and RANTES release by stimulated airway epithelial cells were significantly downregulated by salmeterol. However, while the degree of inhibition observed was similar for the two types of adhesion molecules, a significantly higher downregulation of RANTES release, as compared to IL-8 or GM-CSF release was observed.

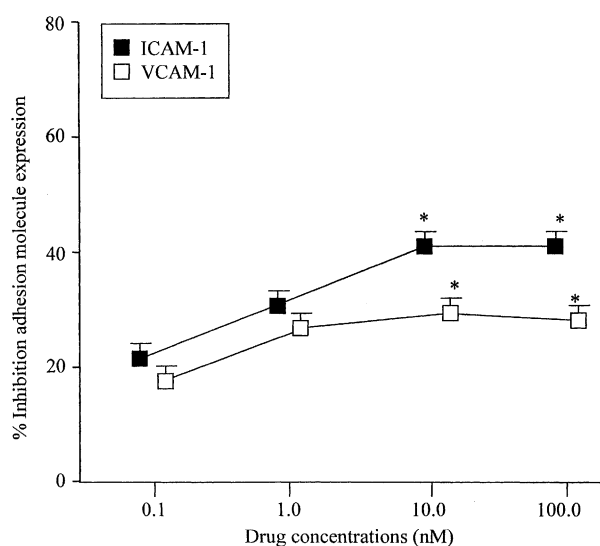
Salmeterol, like the other  $\beta_2$ -adrenoreceptor agonists acts through the interaction with specific seven-transmembrane receptors located in the plasma membranes of a variety of cells, including smooth muscle cells, blood mononuclear cells, fibroblasts and epithelial cells.<sup>5-7</sup> Although some

of the response to  $\beta_2$ -agonists may be mediated through cAMP-independent mechanisms<sup>34</sup> classically the occupation of a  $\beta_2$ -adrenoreceptor by an agonist results in a conformational change that leads to G protein activation and conversion of ATP to cAMP, the second messenger of  $\beta_2$ -adrenoreceptor function.<sup>35</sup> In the airways, cAMP induces smooth muscle relaxation through the activation of the enzyme protein kinase A (PKA) which, besides phosphorylating regulatory proteins involved in the control of muscle tone, lowers the cellular calcium ion (Ca<sup>2+</sup>) concentration.<sup>35</sup> Although PKA may also interfere with the transcription of genes related to synthesis and release of a variety of proteins, including proinflammatory cytokines, the mechanisms

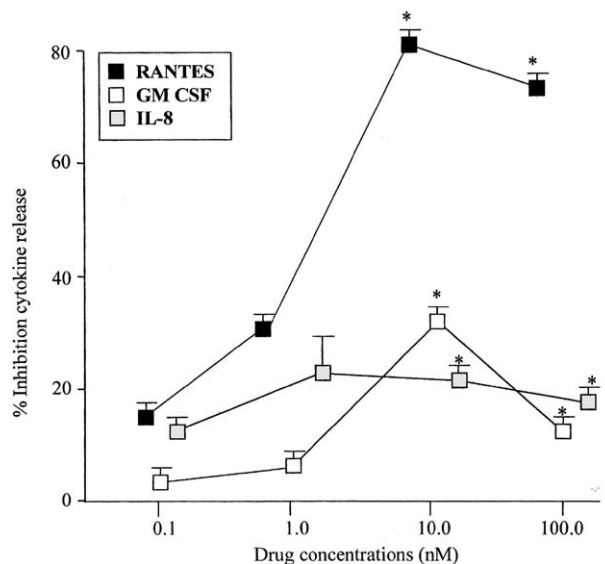




**Figure 2** Changes of IL-8 (A), GM-CSF (B) and RANTES (C) release by human bronchial epithelial cells induced by IL-4, TNF- $\alpha$  or IL-4 + TNF- $\alpha$ . The cytokine concentrations, expressed as pg·mL<sup>-1</sup> are shown on the ordinate, whereas the different culture conditions, CTR, IL-4 (20 ng mL<sup>-1</sup>), TNF- $\alpha$  (20 ng mL<sup>-1</sup>) and IL-4 + TNF- $\alpha$  (20 ng mL<sup>-1</sup>) are shown on the abscissa. The data are expressed as mean  $\pm$  SEM, and represent the results of eight experiments \* $P$  < 0.05; \*\* $P$  < 0.01; as compared to unstimulated cell cultures.



**Figure 3** Effects of different concentrations of salmeterol on the IL-4 + TNF- $\alpha$ -induced ICAM-1 (■) and VCAM-1 (□) expression on human bronchial epithelial cells. The salmeterol induced-inhibition of ICAM-1 and VCAM-1 expression is shown on the ordinate as percentage whereas the different concentrations (0.1 to 100 nM) of the drug are reported on the abscissa. The data are expressed as mean  $\pm$  standard error of the mean, and represent the results of eight experiments \* $P$  < 0.05, as compared to control cell cultures grown in the absence of the drugs.



**Figure 4** Effects of different concentrations of salmeterol on the IL-4 + TNF- $\alpha$ -induced IL-8 (□), GM-CSF (□) and RANTES (■) release by human bronchial epithelial cells. The salmeterol induced-inhibition of IL-8, GM-CSF and RANTES release is shown on the ordinate as percentage whereas the different concentrations (0.1–100 nM) of the drug are reported on the abscissa. The data are expressed as mean  $\pm$  standard error of the mean, and represent the results of eight experiments \* $P$  < 0.05, as compared to control cell cultures grown in the absence of drugs.

regulating non-muscle relaxant cell functions are not fully understood. There is however convincing evidence that, at least for inflammatory and immunoeffector cells,  $\beta_2$ -adrenoreceptor-induced inhibition of cytokine release is strictly related to the increase in intracellular cAMP levels.<sup>10,36</sup> As demonstrated in the present paper for airway epithelial cells, huge differences in the degree of  $\beta_2$ -adrenoreceptor-induced inhibition on cytokine release by mononuclear cells were detected when various interleukins were compared. Indeed, for unknown reasons IL-12 production by monocytes and dendritic cells is effectively inhibited by  $\beta_2$ -agonists, while in the same experimental system IL-1 $\beta$ , IL-1 $\alpha$  and IL-6 are unaffected.<sup>36</sup> Differences between cells at various stages in development or different cell types have been also demonstrated. For example, while peripheral blood monocytes exhibit an inhibition of lipopolysaccharide-induced IL-8 gene transcription and protein secretion when treated with cAMP-elevating autacoid PGE<sub>2</sub>, no effects are seen in alveolar macrophages.<sup>37</sup> Similarly, discrepancies are also found when  $\beta_2$ -adrenoreceptor agonist-mediated downregulation of surface molecule expression by different cell types are compared. Indeed, while salmeterol and fenoterol were effective in downregulating adhesion molecules on monocytes, airway epithelial cells and fibroblasts<sup>9,16,38</sup> no inhibition of the PAF-induced CD11 upregulation on eosinophils when the cells were incubated with procaterol was observed.<sup>39</sup> The data here reported, on a significant "downregulating" activity of salmeterol on airway epithelial cell functions are partially in disagreement with the results of previous studies. Indeed, while fenoterol, a short-acting  $\beta_2$ -adrenoreceptor agonists, effectively inhibited ICAM-1 expression on airway epithelial cells,<sup>16</sup> contrasting results were demonstrated on IL-8, RANTES and GM-CSF release when bronchial epithelium was exposed to other two short-acting  $\beta_2$ -adrenoreceptor agonists, salbutamol and procaterol.<sup>17,18</sup> More recently, in a "similar" in vitro systems, looking at TNF- $\alpha$ -stimulated bronchial epithelial cells, was observed an inhibitory effect of formoterol on GM-CSF release, at levels comparable to those described here with salmeterol, which however was associated with an enhancement of IL-8 release.<sup>19</sup>

These conflicting results may be related to a variety of factors that include differences in experimental protocols,  $\beta_2$ -adrenoreceptor polymorphism genotype of the donors<sup>40</sup>, characteristics of the compound tested and the stimulus used to activate the cells. In addition, although in the same subject,  $\beta_2$ -adrenoreceptors appear to be similar in the cells of different tissues,<sup>41</sup> cellular responses

are dependent on cell types and may also vary between asynchronous cells, at different stages of the cell cycle.<sup>42</sup>

In conclusion, the present report indicates that salmeterol, at concentrations similar to those reached at the airway levels using the drug by inhalation<sup>9</sup>, is effective in downregulating some pro-inflammatory epithelial cell functions.

A characteristic of many membrane-associated receptors is desensitization after high dose or repeated exposure to agonists and  $\beta_2$ -adrenoreceptors on inflammatory cells appear to become rapidly uncoupled from their intracellular signalling pathways and to eventually lost from the cell surface.<sup>1,2</sup> This may be true also for airway epithelial cells. Indeed, heterogeneity in  $\beta$ -adrenergic receptor kinase expression (the enzyme inducing  $\beta_2$ -adrenoreceptor phosphorylation, i.e. inactivation) seems to account for cell-specific desensitisation of  $\beta_2$ -receptor and it has been shown that the level of  $\beta$ -adrenoreceptor kinase mRNA in airway smooth muscle cells are approximately 20% of that in bronchial epithelial cells.<sup>43,44</sup>

Therefore, the in vitro downregulation of proinflammatory functions of airway epithelial cells by salmeterol may not be clinically relevant to the regular treatment in clinical practice. However, the result of the present paper, together with other in vitro data, may possibly explain some acute clinical "anti-inflammatory" activity of  $\beta_2$ -adrenoreceptor agonists,<sup>45,46</sup> not confirmed with the regular prolonged use of these compounds.<sup>47,48</sup>

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